IDENTIFICATION OF Ca^{2+}-BINDING SUBUNIT OF MYOSIN LIGHT CHAIN KINASE FROM SKELETAL MUSCLE WITH MODULATOR PROTEIN

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1. Introduction

The protein present in various vertebrate tissues as smooth muscle, adrenal medulla, brain and platelets, previously identified with troponin C, has been found [1,2] to be identical with modulator protein of cyclic nucleotide phosphodiesterase. This protein was also found in skeletal and cardiac muscles, i.e., in the tissues which contain troponin C. Most of modulator protein was present in the cytosol of all tissues studied but part of it was bound to the structural insoluble proteins [1].

The 18 000 dalton skeletal myosin light chain (P-light chain) is phosphorylated by a specific Ca^{2+}-dependent kinase [3,4]. When this work was in progress, this kinase was reported [5] to consist of two subunits, one of which is a 20 000 dalton Ca^{2+}-binding protein. We present here evidence that this Ca^{2+}-binding protein is identical with the modulator protein of cyclic nucleotide phosphodiesterase and that this protein is necessary for Ca^{2+}-dependent skeletal myosin light chain kinase activity.

2. Materials and methods

Myosin was prepared from rabbit skeletal muscle as in [6]. Minced fresh muscle were extracted with Guba-Straub solution for 15 min, then centrifuged at 4500 × *g* for 15 min. Supernatant was diluted 10-fold, the precipitated myosin was collected by centrifugation and dissolved in 0.5 M KCl. KCl concentration was lowered to 0.3 M and the solution was ultracentrifuged at 75 000 × *g* for 1 h. Supernatant was used for further studies.

The activity of myosin light chain kinase was determined by the appearance of the phosphorylated form of myosin P-light chain detected by means of urea–polyacrylamide gel electrophoresis [3]. Myosin was incubated in 50 mM Tris–HCl (pH 7.5), 0.12 M KCl, 10 mM $MgCl_2$, 2 mM ATP and either 0.1 mM $CaCl_2$ or 1 mM EGTA at 37°C. After 15 min the reaction was stopped by addition of urea to 6 M and the samples analysed by polyacrylamide gel electrophoresis on 8.0% gel, essentially according to [7], with Tris–glycine buffer, pH 8.6, in the presence of 5 M urea. Polyacrylamide gel electrophoresis with SDS was performed according to [8]. Modulator protein and cyclic nucleotide phosphodiesterase were isolated from brain as in [9], and phosphodiesterase activity was measured as in [10]. Troponin C was prepared as in [11].

3. Results and discussion

In agreement with [3] we have found that conventional preparations of myosin from skeletal muscle contain an endogenous kinase which in the presence of Ca^{2+} and ATP phosphorylates myosin P-light chain. The extent of phosphorylation was demonstrated in the polyacrylamide gel electrophoresis in the presence of urea, under the conditions when the phosphorylated form of P-light chain migrates faster than the non-phosphorylated one (fig.1B). During incubation of

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl-ether) *N,N'*-tetraacetic acid; SDS, sodium dodecyl sulphate

myosin with ATP in the absence of Ca^{2+} phosphorylation did not occur (fig.1A). In some control experiments phosphorylation was also followed by measurement of incorporation of ^{32}P from [γ-^{32}P]ATP. The kinase activity seems to be bound to myosin relatively tightly, but it can be released from myosin with EDTA. For this purpose myosin in 0.3 M KCl was precipitated by dilution to a low ionic strength (μ 0.03) with a buffer containing 2 mM Tris–HCl, (pH 7.5) and 2 mM EDTA. Supernatant (EDTA-supernatant) contained whole activity of the light chain kinase. Incubation of EDTA-treated myosin in the presence of Ca^{2+} and ATP did not lead to phosphorylation of P-light chain (fig.1C), and the phosphorylation again took place after addition of EDTA-supernatant to this system.

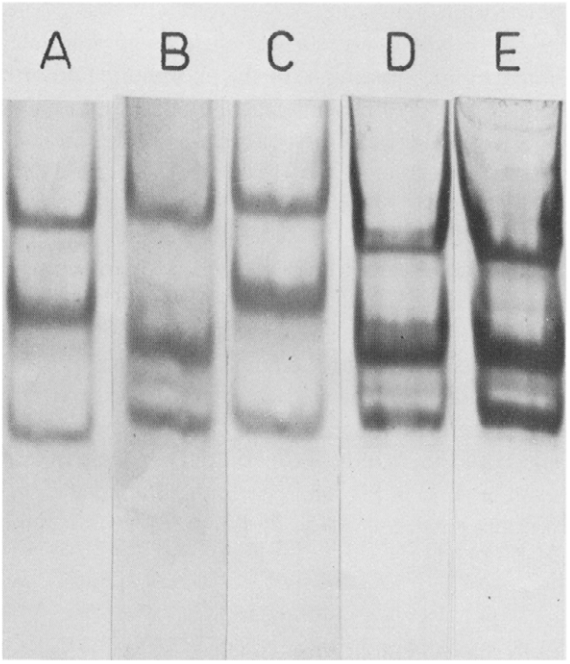

Fig.1. Phosphorylation of skeletal muscle myosin P-light chain. Conditions of phosphorylation and urea–polyacrylamide gel electrophoresis are described in section 2. (A) myosin after incubation with ATP in the presence of EGTA; (B–E) myosin after incubation with Ca^{2+} and ATP: (B) untreated myosin; (C) EDTA-treated myosin; (D) EDTA-treated myosin with fractions (i) and (iii) from DEAE–Sephadex column added; (E) EDTA-treated myosin with fraction (i) from DEAE–Sephadex column and purified brain modulator added.

EDTA-supernatant contained a protein which in the absence of Ca^{2+} migrated in urea–polyacrylamide gel electrophoresis much faster than other proteins and disappeared in the presence of Ca^{2+} (fig.2A). A protein which behaved identically was found in the homogenates of various tissues [1]. It was also found that this protein could be either troponin C or modulator protein, since both of them showed in urea–polyacrylamide gel electrophoresis unusually high mobility in the absence of Ca^{2+} and were bound in the presence of Ca^{2+} to some slowly migrating protein(s) [1].

The results presented in fig.2 indicated that EDTA-supernatant also contained a protein exhibiting the properties typical for troponin C and modulator protein and, moreover, that this protein in the presence of Ca^{2+} formed a complex with other protein(s).

EDTA-supernatant in 30 mM KCl was fractionated on DEAE–Sephadex column in the presence of EDTA. Three fractions were collected:

(i) Not retarded under these conditions.
(ii) Eluted at 0.2 M KCl.
(iii) Eluted at 0.7 M KCl.

None of these fractions alone was able to phosphorylate P-light chain of EDTA-treated myosin. However, the light chain kinase activity was restored when both fraction (i) and (iii) were added to the EDTA-treated myosin. Under these conditions in the presence of Ca^{2+} and ATP the phosphorylation of P-light chain was easily observed (fig.1D). Fraction (iii) contained a protein whose mobility in urea–polyacrylamide gel electrophoresis was higher in the presence of Ca^{2+} than in its absence (fig.2C), a property also shared by troponin C and modulator protein.

In contrast to modulator protein troponin C is at similar concentrations unable to stimulate phosphodiesterase activity [1]. Fraction (iii) activated cyclic nucleotide phosphodiesterase in the presence of Ca^{2+} (table 1) and, moreover, could be replaced in the whole reconstituted phosphorylating system by modulator protein isolated from brain (fig.1E), but not by troponin C at the same concentrations.

All these data indicate that fraction (iii) of EDTA-supernatant contains modulator protein and that this protein is necessary for the activity of skeletal myosin light chain kinase.

In order to obtain a direct proof that modulator

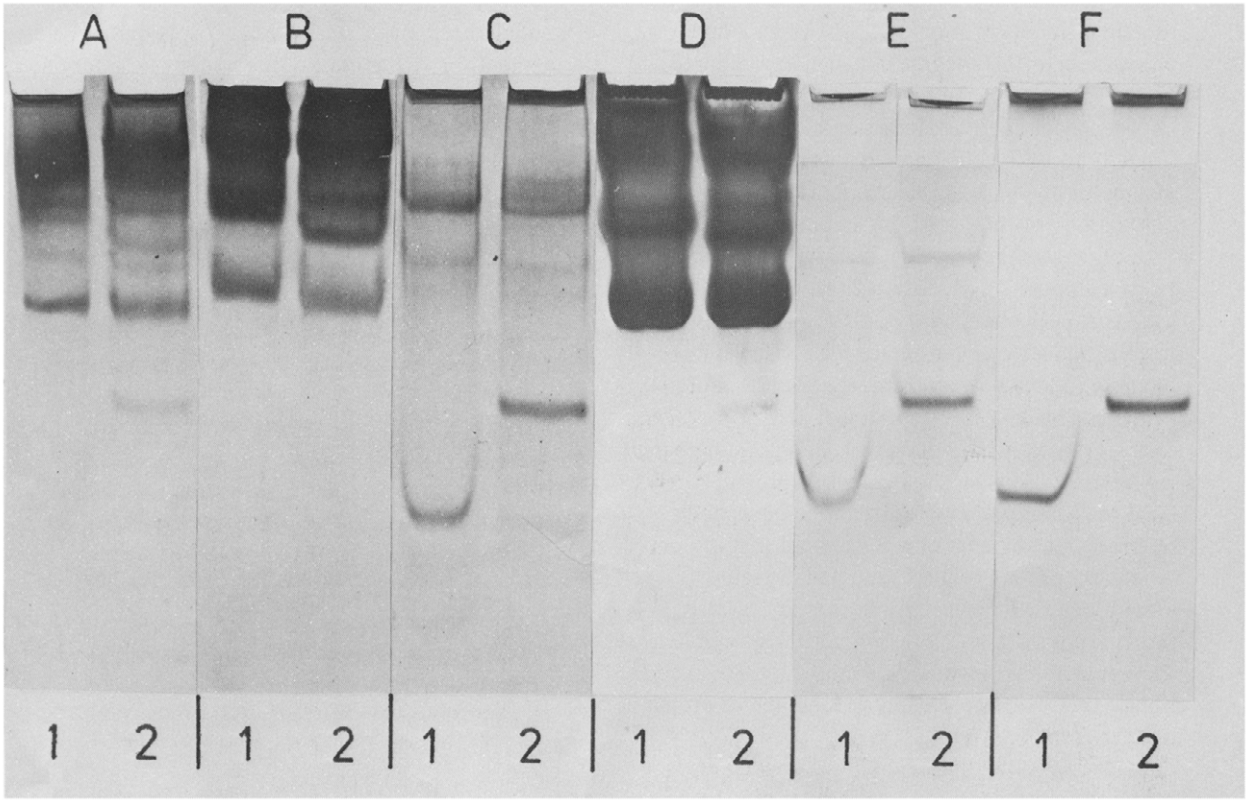

Fig.2. Urea–polyacrylamide gel electrophoresis of crude muscle myosin light chain kinase and its fractions. (A) EDTA-supernatant; (B) fraction (i) from DEAE–Sephadex column; (C) fraction (iii) from DEAE–Sephadex column; (D) crude kinase preparation (fraction 2 in [5]); (E) peak eluted at 0.35–0.45 M KCl from DEAE-cellulose column from fraction 2; (F) brain modulator protein purified according to [9]. Conditions of urea–polyacrylamide gel electrophoresis as described in section 2. Sample 1, 0.1 mM $CaCl_2$ present; Sample 2, 1 mM EDTA present.

Table 1
Effect of the fractions of skeletal muscle myosin kinase on the activity of cyclic nucleotide phosphodiesterase

Additions	Amount added	Phosphodiesterase act. (μmol P_i × mg prot.$^{-1}$ × min^{-1})
1. None	–	0.07
2. Brain modulator protein	1.2 μg	0.65
3. EDTA-supernatant fractionated on DEAE-Sephadex column:		
(a) fraction (i)	150 μg	0.07
(b) fraction (iii)	15 μg	0.56
4. Peak eluted at 0.35–0.45 M KCl from DEAE-cellulose column from fraction 2 in [5]	4 μg	0.63

protein is a subunit of myosin light chain kinase a preparation of this enzyme obtained according to [5] was analysed. Fraction 2 in [5], which reveals a Ca^{2+}-dependent light chain kinase activity, contained similarly to EDTA-supernatant a protein which in urea polyacrylamide gel electrophoresis appeared only in the presence of EGTA and migrated faster than other proteins (fig.2D). Fraction 2 was further fractionated on DEAE-cellulose column according to [5]. A peak eluted at 0.35–0.45 M KCl was found to consist chiefly of a protein which changed its mobility in urea polyacrylamide gel electrophoresis depending on concentration of Ca^{2+} (fig.2E), similarly to protein present in fraction (iii) of EDTA-supernatant from DEAE–Sephadex column. This protein stimulated the activity of cyclic nucleotide phosphodiesterase to the extent comparable with that of brain modulator protein (table 1) and had the same mobility in SDS–gel electrophoresis as the latter protein (not shown). All the properties, as well as the fact that the protein present in the kinase preparations can be fully replaced by the modulator protein isolated from brain, suggest very close similarity if not identity between the subunit of skeletal myosin light chain kinase and brain modulator protein.

In parallel studies [12] one of two subunits of smooth muscle myosin light chain kinase appeared also identical with the modulator protein. Our preliminary studies [13,14] seem to suggest that preparations of actomyosin from brain, platelets and *Physarum* also contain modulator protein.

All these observations may imply that the same or very closely related protein, known so far to be modulator of cyclic nucleotide phosphodiesterase metabolism, is a subunit of the kinase phosphorylating light chain of myosin regardless of its origin and that this protein confers Ca^{2+} sensitivity of motile phenomena in various non-muscle systems.

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